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Award Number: DAMD17-00-1-0580

TITLE: Controlof Alcoholism-Related Folate Deficiency by Regulation of Urinary Folate Excretion

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REPORT DATE: October 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (1 Oct 01 - 30 Sep 02)

4. TITLE AND SUBTITLE
Controlof Alcoholism-Related Folate Deficiency by Regulation of Urinary Folate Excretion

5. FUNDING NUMBERS
DAMD17-00-1-0580

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Health-related consequences of heavy drinking include nutritional deficiencies. Folate deficiency occurs from multiple causes including increased urinary folate excretion. Ethanol decreases the renal reabsorption of folate leading to increased excretion. The main aim of this research is to determine the mechanism by which ethanol decreases folate transport by the kidney. Initially, the effects of ethanol on expression of the two renal transport proteins, the folate receptor (FR) and the reduced folate carrier (RFC), were determined in vivo and in vitro. 5 day treatment with ethanol increased the content of both transporters in cultured human proximal tubule cells, while 14 day feeding of rats with ethanol-containing diets increased the content of both transporters in isolated rat kidney membranes; the RFC was increased to a greater extant than was the FR. Repeated ethanol treatment may increase transporter content to counter the acute ethanol-induced decrease in folate uptake, hence to restore folate homeostasis. Preliminary studies are using fluoroscein-methotrexate as a ligand for the RFC to examine the hypothesis that ethanol has its effects via the RFC pathway. Studies will also use transport kinetic and pathway inactivation studies to confirm which transporter is affected by ethanol.

14. SUBJECT TERMS
alcoholism, chronic ethanol abuse, urinary folate excretion, folate deficiency, folate transport proteins, cultured kidney cells

15. NUMBER OF PAGES
44

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Recent surveys have shown that military personnel are more likely to drink heavily (≥ five drinks at least once per week) than civilian controls (1). Although overall alcohol use has declined, nearly 20% of military personnel engage in heavy drinking and alcohol-related consequences continue to be a problem. Health-related consequences include alcohol-induced nutritional deficiencies such as deficiency of the vitamin folic acid (2), which can lead to megaloblastic anemia, to neural tube birth defects or possibly to atherosclerotic disease. In animals, ethanol treatment markedly increases urinary folate excretion by decreasing renal folate reabsorption(3,4), thereby contributing substantially to the folate deficiency. These effects are observed at blood levels in the range of 150-300 mg/dl (5,6), which are often reached in heavy drinking humans. Acute ethanol administration decreases the apical (AP) transport of the physiologic folate, 5-methyl-tetrahydrofolate (5M), by normal human proximal tubule (HPT) cells in culture. The two pathways known to be involved in the AP transport of 5M by cultured proximal tubule cells are the folate receptor (FR) and the reduced folate carrier (RFC) (7). The primary objective of this research is to determine the mechanism by which ethanol alters 5M transport by the proximal tubule cell since urinary folate excretion is primarily regulated by the reabsorption of 5M from the tubular lumen. Our hypothesis is that ethanol decreases the AP-mediated transport of 5M (hence its reabsorption) by decreasing the activity in either the FR or RFC pathway. After either acute or chronic ethanol treatment, 5M transport will be studied in HPT and rat PT cells, cultured on membrane filter inserts to maintain distinct AP and basolateral (BL) compartments (8). The major aims will be to determine which pathway is affected by ethanol using a combination of kinetic studies, pathway inactivation studies and immunoblotting studies of the content of each of the proteins. Hence, these studies will provide mechanistic information that is needed to design interventions to reduce the morbidity from the folate deficiency that results from chronic heavy drinking.

BODY

Technical Objective 1. **To characterize the effects of acute and chronic ethanol on bidirectional renal folate intracellular transport.** In the Statement of Work, this objective was to be accomplished in Years 1 and 2. Previous studies of folate uptake by HPT cells have shown that a 1 hr exposure of HPT cells to ethanol in concentrations > 100 mg/dl significantly decreases the AP-directed transport of 5M, without affecting its basolateral transport (details of these results are in the manuscript in the Appendix). As a follow-up to these studies, we examined the effects of 1 hr ethanol exposure on the AP uptake of [^{14}C]-α-methylglucoside (αMG), a marker for AP-directed glucose transport (8). At concentrations up to 300 mg/dL, ethanol did not affect αMG uptake by HPT cells, although ethanol at 500 mg/dL did significantly decrease its uptake by about 30%. These results suggest that ethanol appears to specifically reduce folate transport by HPT cells at concentrations between 100 - 400 mg/dL, but reduces uptake of both folate and glucose at high concentrations.

Similar studies that examined the effects of 5-day pretreatment of HPT cells with ethanol have now been completed and details are included in the same manuscript. Specific AP cellular transport of [^{3}H]-5M in pretreated HPT cells, expressed per mg cellular protein, was not significantly altered at any concentration of ethanol as compared to control (Figure 1). The fact that acute ethanol treatment significantly reduced specific AP transport, while repeated ethanol treatment

did not, might be explained by the results in Technical Objective 2, where repeated ethanol treatment increased the content of folate transport proteins in HPT cell membranes. The increase in folate transporters after 5 days would be expected to partially counteract the acute reduction in transport, which could explain the minimal effect of 5-day ethanol treatment on AP-mediated transport of folate. The protein content of the HPT cell cultures treated with ethanol for 5 days was significantly decreased by 24, 27, and 30% at 300, 400, and 500 mg/dl, respectively as compared to control, indicating a lower number of cells per well at the high ethanol concentrations. When total folate uptake per well was computed, without normalizing the data to amount of protein per insert, ethanol at 400 and 500 mg/dl produced a decrease in both AP binding and cellular transport of 5-M by about 20-30%, a magnitude similar to the reduction in cellular protein. The reduction in total uptake, but not in uptake per mg protein, would be a result of the lower amount of protein (reduced number of cells per insert). Despite the apparent loss of cells in these repeated ethanol studies, ethanol per se did not induce cell death. In one HPT isolate at the end of the 5 day exposure, the cells were treated with ethidium homodimer, a dye that is taken up only by cells with damaged membranes and is used as a measure of cytotoxicity (9). No increases in ethidium homodimer fluorescence was noted indicating that ethanol at concentrations up to 500 mg/dl did not affect cellular viability.

Technical Objective 2. **To characterize the interaction of ethanol with the renal folate receptor (FR), as a mechanism by which urinary folate excretion could be affected.**
Technical Objective 3. **To characterize the effects of ethanol on the renal reduced folate carrier (RFC) activity, as a mechanism by which urinary folate excretion could be affected.** These objectives are discussed together since they are being tested simultaneously, with the Statement of Work stating that the effects of ethanol on renal FR and RFC expression in cells and in vivo will be tested in Years 1 and 2. We have made significant progress on these studies this past year.

Effect of repeated ethanol treatment on FR and RFC expression in HPT cells. Because acute treatment of HPT cells with high concentrations of ethanol decreases AP-mediated folate uptake, we subsequently measured the effects of repeated ethanol on the content of the two folate transporters in the AP membranes of HPT cells. HPT cells were seeded into tissue culture flasks (75 cm^2) and grown to near confluency, when the growth media were changed in respective flasks to the same media containing ethanol in concentrations of 0, 100, 300 and 500 mg/dL (0, 22, 65 and 109 mM). Cells were exposed to ethanol for five days, by replacing the media daily to ensure continued exposure to the expected ethanol levels. Particular care was taken to minimize the potential cell loss, as noted in the above 5 day studies, by extremely gentle treatment of flasks during the media changes. Light microscopic observation after 5 days revealed no differences in cell content in the flasks, nor in the total protein content of the recovered cells. These results indicated no cell loss during these studies. After 5 days, the cells were removed from the flasks by scraping into "Sucrose" buffer (0.25 M sucrose, 10 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.0, containing 1 mM aminoethylbenzenesulfonylfluoride (AEBSF), 100 μM leupeptin and 10 μM bestatin as protease inhibitors). Cells were homogenized in glass homogenizers and AP membrane fractions were obtained by differential centrifugation (10). The resulting pellets were resuspended in sucrose buffer containing fresh protease inhibitors and stored frozen for analysis. The membrane fractions were thawed, solubilized in 2% SDS and subjected to SDS-PAGE prior to transfer to PDVF membranes for immunoblot analysis (11). After blocking to minimize nonspecific binding, the membranes were treated with primary antibodies to either the FR (a rabbit polyclonal anti-human placental FR preparation) or the RFC (a rabbit polyclonal anti-human recombinant RFC preparation). Membranes

were then treated with anti-rabbit secondary antibodies and the complexes were detected by enhanced chemiluminesence and quantitated by computer-assisted densitometry.

Reproductions of the original blots from two isolates are shown in Figure 2, along with the resulting densitometry scans. A summary of the results from 4isolates is in Figure 3. Repeated ethanol treatment dose-dependently increased the content of both the RFC and the FR in the AP membranes of HPT cells, with significant effects at higher than 100 mg/dL. Ethanol appeared to increase the content of the RFC (4-5 fold) to a much greater extent than its effect on the FR (2 fold). This up-regulation of both folate transport pathways might be expected as an adaptive response to the previously-shown decrease in folate uptake by acute ethanol treatment. That is, the cells increase the content of the folate transporters to counteract the initial decrease in activity of the folate uptake pathways due to ethanol exposure. This adaptation returns the amount of folate uptake towards normal. These studies also suggest that the ethanol may primarily affect the RFC pathway, which has led to our initial studies below that are examining the inhibitory effects of ethanol on RFC-mediated transport.

Effect of repeated ethanol treatment on FR and RFC expression in rat kidneys in vivo. Acute ethanol treatment of rats increases urinary folate excretion about 6-fold (3), while chronic ethanol treatment of rats also increases urinary folate excretion within two weeks, but by only two-fold levels (5). In order to examine whether ethanol affects urinary folate excretion by altering the expression of the RFC or FR in the AP membranes of the rat kidney in vivo, we conducted the following study. Sixteen male Sprague-Dawley rats (about 250-300 g) were acclimated to control liquid diets (Lieber-DeCarli formula (5)) for a period of 4 days, then were divided into two groups of 8 rats, fed either an ethanol diet (36% of calories) or a control diet (isoenergic carbohydrate to replace ethanol). Each control animal was fed its diet in a pair-fed manner to a corresponding ethanol rat (restricted to the previous day's consumption of its pair) in order to maintain consistent nutrient intake between groups. Food consumption and rat weights were obtained daily. On Day 13, rats were removed from their home cages and placed in metabolic chambers for 24 h, during which rats had continued access to their diets. Urine was collected at 8 h intervals into tubes that contained 2-mercaptoethanol and were wrapped in foil to minimize oxidation of folates. Collections were combined for a 24 h sample, then stored frozen for folate analysis by microbiological assay and ethanol analysis by gas chromatography. The rats were returned to their home cages for the 14th day. On the morning of the 15th day, rats were sacrificed by decapitation and the kidneys were rapidly removed and stored at -20° C. The cortex was removed from the stored, partially thawed kidneys by careful excision, then was homogenized in sucrose buffer (as above, except containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as the protease inhibitor). The AP membranes were recovered by differential centrifugation (10), solubilized in SDS and prepared by immunoblotting to measure FR and RFC content, as described above for the HPT cell membranes.

On day 13 of the study, the mean urinary ethanol concentration in the ethanol-fed rats was 181 ± 40 mg/dL, indicating substantial exposure of the rats to ethanol over the 2 wk feeding period. The mean urinary folate excretion in the ethanol-fed rats was 9.0 ± 2.3 pmol/24 h, which was significantly greater than that in the controls (2.5 ± 0.5), confirming the effect of ethanol to increase urinary folate. Reproductions of the original blots and the corresponding densitometry scans are summarized in Figure 4. The results indicate that the 2 week ethanol treatment increased the content of both the FR and the RFC in the AP membranes of rat kidneys, with a somewhat larger effect on the RFC. Similar to the HPT cells, these results suggest that the rat kidney responds to increase the amount of the folate transporters as a way to counteract the acute effect of ethanol (which is to

decrease the reabsorption of folate from the lumen leading to increased urinary folate excretion). The fact that acute ethanol increases excretion about 6-fold, while chronic treatment increases it only about 2-3-fold indicates that one would expect some compensation to have occurred in the chronic animals. Our results suggest that an up-regulation of the transporters is the mechanism for this compensation. Similarly to the in vitro studies, the RFC seems to be increased somewhat more than the FR. Also, the upregulation in vivo was not as large as that observed in vitro. The larger upregulation in vitro was observed at ethanol concentrations in the range of 300-500 mg/dL, while the rats probably were exposed to ethanol in the 150-250 mg/dL range. The in vitro studies showed that the upregulation was dose-dependent, so a lesser effect at the lower concentrations in vivo would normally be expected.

Mechanism for the acute effect of ethanol on renal folate transport. The repeated ethanol studies indicated that ethanol affects the content of the RFC in the renal apical membranes to a greater extent than the content of the FR, so one might conclude that the RFC is the more important pathway for folate uptake in the PT cells. Hence, we have initiated studies of the RFC-mediated uptake by HPT cells using a new method of assessment. Methotrexate (MTX) is a reduced folate analog that is preferentially taken up by the RFC, with little affinity for the FR. Recent studies have demonstrated the usefulness of the fluorescein-MTX conjugate (FL-MTX) as a surrogate substrate for MTX transport by the RFC in human leukemic cell lines (12). Our initial studies have examined the AP uptake of FL-MTX by HPT cells using confocal microscopy as a direct and sensitive measure of MTX (folate) transport by the RFC.

HPT cells were grown to confluency in 24 well plates, washed, then treated with FL-MTX (0, 0.5, 1, 2 and 4 μM) in PBS for 45 min at room temperature. The cells were directly observed using the Bio Rad Radiance 2000 Confocal microscope and viewed through a 40X CF infinity plan fluor objective. Excitation was provided by the 488 nm line of an argon laser. A 515/30-nm filter and a 545/40-nm dichroic filter were used. The images were viewed using Bio-Rad's Lasersharp-I, which allows 32-bit image acquisition and 3D image analysis. The results in Figure 5 indicate that the cellular absorption of FL-MTX is directly proportional to concentration of FL-MTX. Next, to 24 well plates containing HPT cells, FL-MTX (1-4 μM in PBS) and different concentrations of ethanol (500 to 1000 mg/dl) were added and the cellular accumulation of FL-MTX was measured using confocal microscope. A qualitative assessment of the ethanol study seemed to indicate that ethanol at these very high concentrations decreased the apparent accumulation of FL-MTX by HPT cells. Further analysis of the results in a quantitative manner will be done subsequently. If true, the data would indicate that ethanol affects folate transport through the RFC pathway.

KEY RESEARCH ACCOMPLISHMENTS

- Ethanol treatment of HPT cells for 1 hr inhibits the apical transport of folate, but not that of glucose analogs, at concentrations in the range 100-400 mg/dL, suggesting a specific effect on folate transport. In contrast, repeated exposure of cells to ethanol for 5 days does not significantly decrease apically-mediated folate transport, probably due to the upregulation of folate transport pathways by the repeated exposure.

- Exposure of HPT cells to ethanol for 5 days up-regulates both folate transporters, the FR and the RFC. This effect is concentration related, with effects appearing at levels > 100 mg/dL. The effects on the RFC are greater than the effects on the FR. The increase in folate

transporter content in the cell membranes may be a compensatory reaction to overcome the ethanol-induced decrease in folate transport into the cells.

- Treatment of rats with ethanol in the diet for 14 days increases the FR and RFC levels in the AP membranes of the rat kidneys, with a somewhat greater effect on the RFC. This increase in transporter content should counteract the decrease in folate reabsorption (increase in urinary excretion) that occurs due to acute ethanol and may represent an adaptive response.

- Initial studies have shown that HPT cells dose-dependently take up fluorescein-MTX, a specific ligand for the RFC. Treatment with ethanol , suggesting that ethanol inhibits folate reabsorption (apical transport) by the RFC.

REPORTABLE OUTCOMES

Abstracts

1. Romanoff RL, McMartin KE. Repeated ethanol upregulates folate transport in the kidney. Alcoholism Clin Exp Res 26: 144A, 2002. Presented at the Research Society on Alcoholism meeting, San Francisco, CA, July 3, 2002.

2. KE McMartin, RL Romanoff. Renal folate transport proteins are regulated by exposure to repeated ethanol and to nutritional deficiency, presented at the FASEB Summer Research Conference on Folate, Vitamin B12 and One Carbon Metabolism, Snowmass, CO, August 4, 2002 (unpublished).

Thesis

1. Romanoff RL, Repeated and sub-chronic ethanol exposure upregulates renal folate transporter proteins, MS thesis, Louisiana State University Health Sciences Center - Shreveport School of Graduate Studies, June 2002.

Manuscript

1. Romanoff RL, Ross DM, McMartin KE, Inhibition of renal folate transport by acute ethanol and up-regulation of renal folate transport proteins by repeated ethanol exposure in rats and human cells, manuscript in final preparation (draft version in appendix - needs final approval from all authors prior to submission).

CONCLUSION

We have made reasonable progress towards the technical objectives outlined in the Statement of Work. We expected to measure the effects of ethanol on renal FR and RFC expression in cells and in vivo and we have finished these studies. We expected to characterize the folate transport kinetics after chronic (repeated) exposure and to measure the folate metabolites to assess how ethanol affects these processes in the kidney. The former studies have been completed, but the latter

ones are to be done in year 3, so we are basically on schedule.

Repeated ethanol treatment of kidney cells in culture and chronic ethanol treatment of rats in vivo increases the content of the two folate transport proteins, the FR and the RFC, in the AP membranes of the kidney. Initially this increase in folate transporter content would seem to be counterintuitive since such ethanol treatments are known to decrease the activity of AP-mediated folate transport. The increase in protein content could be explained as an adaptive response by the cell or the organism to counteract the acute decrease in folate uptake. In such a way, the cells react to the initial decrease in folate uptake by up-regulating the overall number of transporters. The increased number of transporters would then act to restore the level of folate uptake towards normal. This hypothesis is backed by previous data on folate excretion - after one ethanol treatment, urinary folate excretion is increased to a much larger extent than after two weeks of ethanol exposure, suggesting an adaptive response.

"So what". Although these studies have produced interesting results, we have not yet determined the mechanism by which ethanol initially produces the decrease in renal cell uptake of folate. Hence, our studies in the coming year will be focussed on determining which pathway, either the FR or the RFC is decreased by ethanol. As originally outlined in the proposal, we will use specific substrates to measure each pathway (such as our initial studies with FL-MTX and confocal microscopy) and also specifically inactivate each pathway with covalent inhibitors prior to the ethanol treatments in order to assess which pathway is affected by ethanol.

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APPENDICES

1. Figures 1 - 5.
2. Copy of Research Society on Alcoholism abstract (Romanoff RL, McMartin KE. Repeated ethanol upregulates folate transport in the kidney. Alcoholism Clin Exp Res 26: 144A, 2002).
3. Copy of manuscript (Romanoff RL, Ross DM, McMartin KE, Inhibition of renal folate transport by acute ethanol and up-regulation of renal folate transport proteins by repeated ethanol exposure in rats and human cells) - it is in draft form and has yet to be submitted because the final form needs to be approved by Dr. Ross who is no longer at LSUHSC. It should be submitted within a couple of weeks.

FIGURE 1. Effect of 5-day ethanol on apically-directed 5-methyltetrahydrofolate uptake by HPT cells. Data represent mean (% of control at 0 ethanol) ± SEM (n = 4). No significant differences were noted between ethanol and controls.

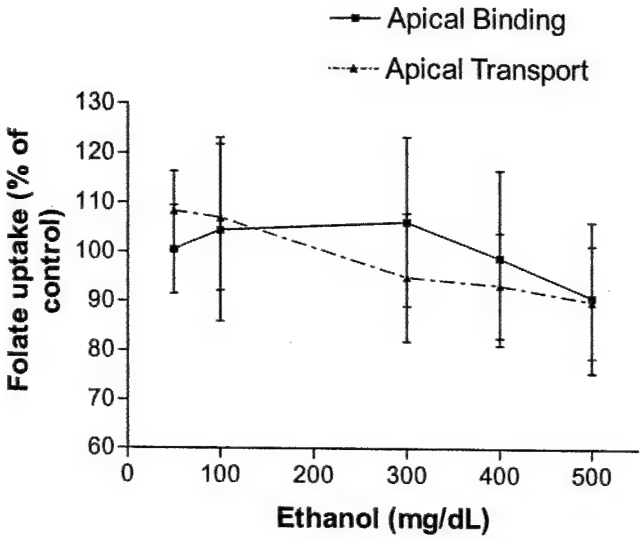


FIGURE 2. Repeated ethanol dose-dependently upregulates folate transport proteins in individual HPT cell isolates. Western blots (n = 3) were performed on HPT isolates - the upper is the RFC (83 kD; 10 μg per lane), the lower is the FR (35 kD; 20 μg per lane). Graphs below represent the optical density (OD) units, normalized to control (0 ethanol blot OD), group mean ± SEM. Bars with letters in common are not significantly different ($p < 0.05$, ANOVA with Tukey's test).

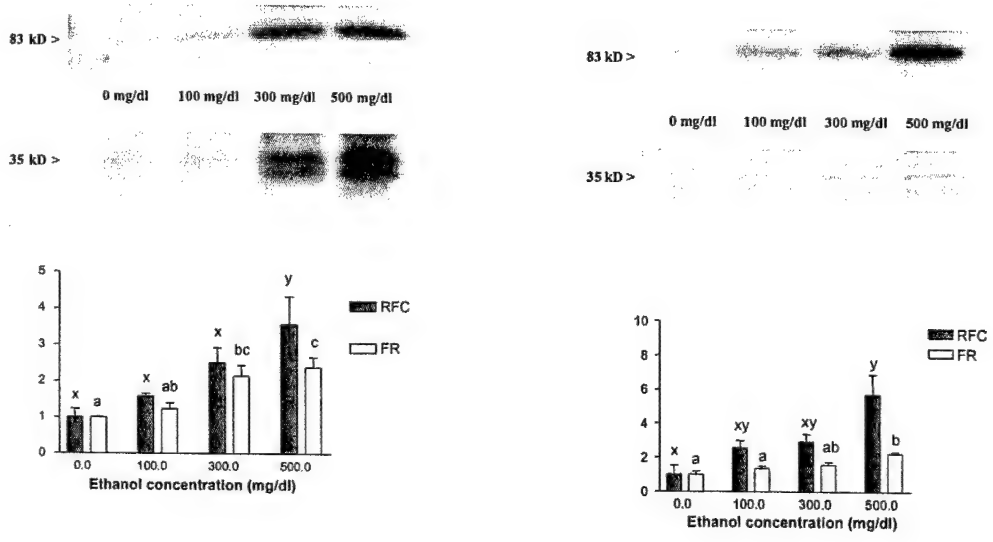


FIGURE 3. Effect of repeated ethanol on folate transport protein expression in HPT cells. OD from Western blots of all isolates exposed to repeated ethanol were combined and normalized to control (0 ethanol). Values are group mean ± SEM (n = 4). Bars with letters in common are not significantly different ($p < 0.05$, ANOVA with Tukey's test).

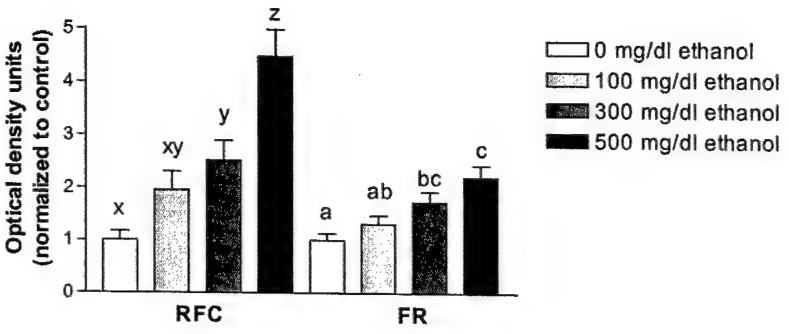


FIGURE 4. Sub-chronic ethanol upregulates folate transport proteins in rat kidneys in vivo. Western blots were performed on isolated renal apical membranes of rats fed diets for 14 days. Upper blots - RFC in 16 rats (83 kD; 10 μg per lane); lower blots - FR in 16 rats (35 kD; 20 μg per lane). "C" = control diet; "E" = ethanol diet pairfed in adjacent wells. Graph below is the OD units, normalized to control (OD of the control diet paired rat). Values represent group mean ± SEM. ** indicates significant difference from control ($p < 0.01$, Student's t-test).

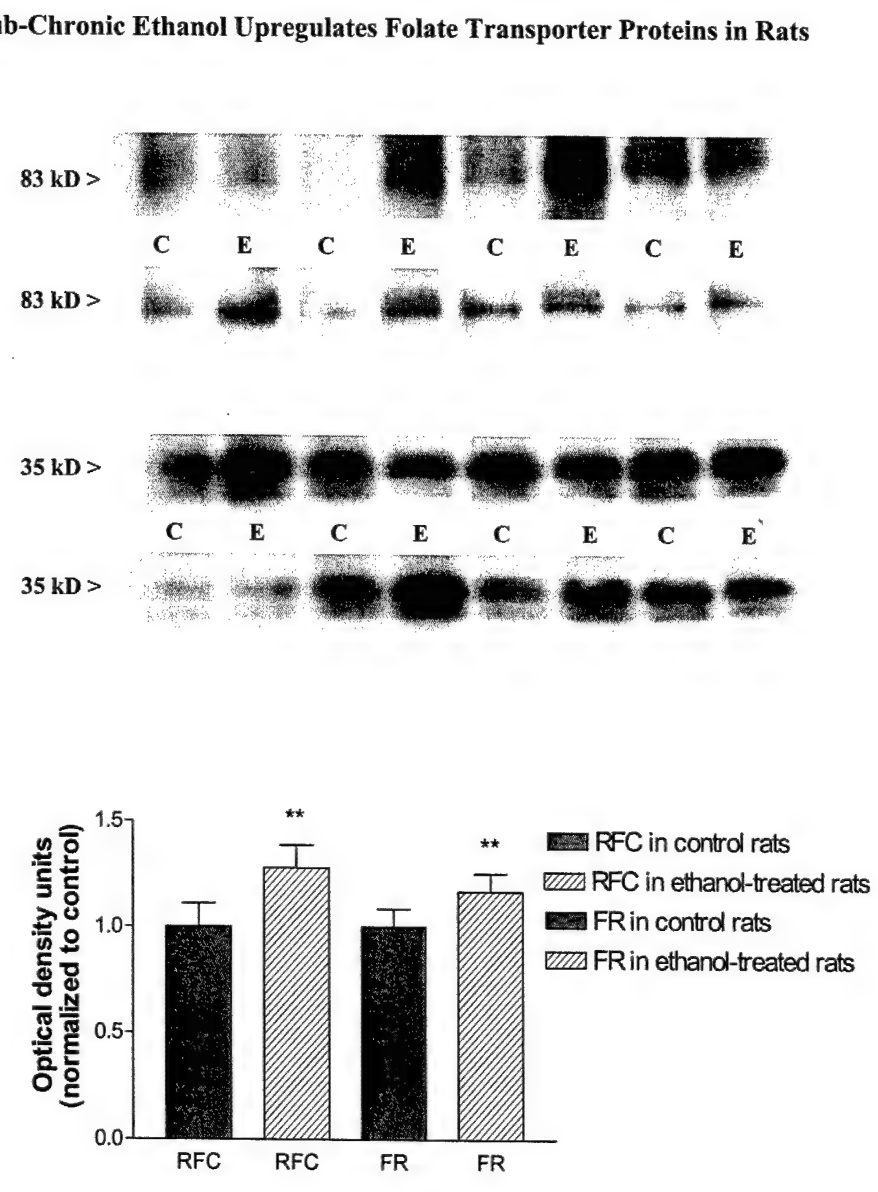
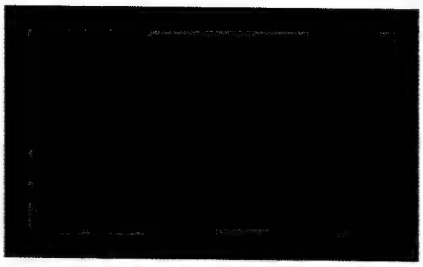
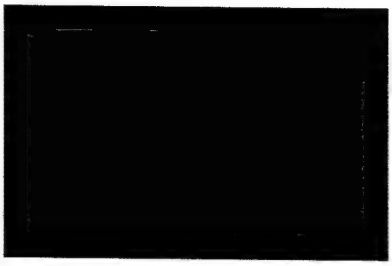


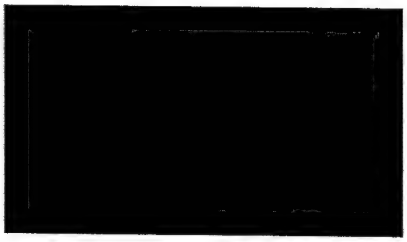
FIGURE 5. FL-MTX transport into HPT cells is directly proportional to the concentrations of FL-MTX as detected by confocal microscopy.



4 μM FL-MTX



2 μM FL-MTX



1 μM FL-MTX

0.5 μM FL-MTX

2. CELL BIOLOGY

A. Membrane biology

835

REPEATED ETHANOL UPREGULATES FOLATE TRANSPORT PROTEINS IN THE KIDNEY

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Alcoholism is an important factor in the development of folate deficiency, as chronic alcohol consumption directly alters folate homeostasis in the body via increased urinary folate excretion. There are two folate transport systems located on the apical brush border membrane of proximal tubule cells in cortical nephrons: the reduced folate carrier (RFC) and the folate binding protein (FBP) or folate receptor (FR). Although the mechanisms of ethanol in increasing urinary folate excretion are unknown, we hypothesize that this increased excretion mainly results from decreased reabsorption. In response to the increased excretion and subsequent decrease in serum folate levels, the transporters may upregulate to maintain folate homeostasis. As an in vitro study, human proximal tubule cells were cultured and repeatedly exposed to various ethanol concentrations (0, 100, 300, 500 mg/dl). The cells were homogenized, and the expression of the FR and RFC transporters was determined by Western blotting. As an in vivo study, male Sprague-Dawley rats were administered ethanol in a liquid diet and compared to control animals. The rats were sacrificed, their kidneys removed and homogenized, and the levels of transporter proteins were examined by Western blotting. The in vitro studies revealed that with increasing ethanol concentrations, the expression of both the FR and RFC transporters increased, with a larger effect observed for the RFC protein. The in vivo study revealed that both folate transporters were upregulated in the ethanol-treated rats as compared to controls, with the increased expression being significant for the RFC protein. These results suggest that the ethanol-induced folate deficiency results in an upregulation of the folate transporters to counteract the acute effects of ethanol in decreasing the reabsorption of folate.

INHIBITION OF RENAL FOLATE TRANSPORT BY ACUTE ETHANOL AND UP-REGULATION OF RENAL FOLATE TRANSPORT PROTEINS BY REPEATED ETHANOL EXPOSURE IN RATS AND HUMAN CELLS

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Running title:

Ethanol and folate transport in the kidney

Research Support:

Supported in part by a research grant from NIAAA and from the US Army Material and Defense Command

ABSTRACT

Background.

Methods

Results

Conclusions

Key words:

INTRODUCTION

Folate is a water-soluble B vitamin essential for cell metabolism and normal growth (Saltman et al., 1987). Deficiency of folate is associated with megaloblastic anemia (Eichner & Hillman, 1971; Halsted, 1980a), neural tube defects in fetuses (Creizel & Dudas, 1992; MRC Vitamin Study Research Group, 1991) and, potentially, atherosclerosis (Brattström et al., 1988; Selhub et al., 1996). The prevalence of folic acid deficiency in chronic alcoholic populations is as large as 87% (Halsted, 1980a). Although the poor diet of alcoholics contributes to their folate deficiency, ethanol directly alters folate homeostasis in the body via the following mechanisms: decreased intestinal folate absorption, increased hepatic retention of folate, and increased loss of folate stores through urinary and fecal excretion (Romero et al., 1981; Tamura et al., 1981; Weir et al., 1985). For chronic alcoholics, even vitamin supplements do not necessarily prevent the depletion of folate (Lindenbaum and Lieber, 1969; Weir et al., 1985).

Increased urinary folate excretion is a principal mechanism for the ethanol-induced folate deficiency. Acute or chronic administration of ethanol results in an increased urinary excretion of folate, which precedes a decrease in plasma folate levels (Tamura and Halsted, 1983; McMartin 1984). In humans as well as rats, acute ethanol ingestion markedly decreases plasma folate levels in 16-20 hours, which then contributes to the development of folate deficiency (Eichner and Hillman, 1973; McMartin, 1984; Paine et al., 1973). In rats, studies have demonstrated that urinary folate excretion is increased 2-fold and 6-fold after chronic (12 wks) and acute (4 hr) ethanol ingestion, respectively, with a subsequent decrease of 5-methyltetrahydrofolate in plasma (McMartin and Collins, 1983; McMartin et al., 1989). This increased urinary excretion was able to completely account for the plasma folate depletion (McMartin, 1984). Urinary folate excretion also increases and contributes to the development of folate deficiency in monkeys chronically consuming ethanol (Tamura and Halsted, 1983) and in ethanol-consuming alcoholics (Russell et al., 1983). The effects of ethanol on urinary folate excretion in acute and chronic studies occur only at high blood ethanol levels, 200-300 mg/dl and 150-200 mg/dl, respectively (McMartin et al., 1989; McMartin et al., 1986). These levels are relevant, as chronic alcoholic populations exhibit folate deficiencies at blood ethanol levels in the 200-300 mg/dl range (Halsted, 1980a). In alcoholics, it is therefore possible that a principal factor in maintaining folate homeostasis is the regulation of urinary folate excretion.

The mechanisms by which ethanol increases urinary folate excretion are unknown. In the kidney, plasma folate is filtered at the glomerulus, and then the excretion of folates in the urine occurs only after the proximal tubule (PT) cells have reabsorbed the majority of folate in the lumen. Experiments *in vivo* have demonstrated that ethanol does not increase the glomerular filtration rate or the amount of folate presented to the kidney (Eisenga et al., 1989a; Eisenga and McMartin, 1987). In contrast, ethanol has a direct effect on the isolated perfused rat kidney (IPRK), where ethanol alters the reabsorption of folate in the PT, resulting in increased excretion (Muldoon and McMartin, 1994). Hence, ethanol's effects appear to result from a decreased reabsorptive transport of folate in the PT.

The roles of folate transporters in mediating the ethanol-induced inhibition of renal folate transport have not been defined. Two folate transporter systems are considered to be responsible for regulating folate homeostasis. These transporters include a glycosylated integral membrane protein, the reduced folate carrier (RFC), and a membrane-associated folate receptor (FR), also referred to as the folate binding protein (Jansen, 1999). The RFC is an anion exchange carrier, similar to the family of 12 transmembrane-domain spanning transporters (Antony, 1996; Jansen, 1999; Kamen et al., 1991). The FR is located in the outer layer of the plasma membrane through a linkage to a glycosylphosphatidylinositol (GPI) anchor (Jansen, 1999). The RFC has been identified in kidney epithelial cells, where it is involved in the transport of folates into the cytosol (Kamen et al., 1991). The FR has also been located in renal PT cells, where it functions to concentrate folates at the apical surface of these cells for internalization, resulting in a net reabsorption of folates from the glomerular filtrate (Rothberg et al., 1990). The RFC and the FR are both proposed to reside in the apical brush border membrane (BBM) of renal PT cells, and an important role has been shown for both the FR and RFC in renal folate transport (Morshed et al., 1997; Sikka and McMartin, 1998).

In order to understand the mechanisms involved in ethanol's effects on urinary folate excretion, it is important to understand ethanol's effects on the transport pathways in both the apical and basolateral membranes of the PT cell, including the roles of the FR and RFC. In studying these mechanisms of folate excretion, a system is needed to distinguish between the apical and basolateral components of PT cells, such as the culturing of human PT (HPT) cells on microporous membrane inserts (Morshed and McMartin, 1997). In the present studies, the HPT cell culture model was used

to determine the effects of acute and repeated ethanol on the apical- and basolateral-directed uptake of 5-methyltetrahydrofolate by the HPT cell. Following the acute and repeated ethanol studies in HPT cells, which examined folate transport, the effects of repeated and sub-chronic ethanol on the folate transporter proteins were investigated in both human (*in vitro*) and rat (*in vivo*) PT cells. The repeated ethanol study in HPT cells was the first experiment to examine the effects of ethanol on the expression of both folate transporter proteins in normal mammalian cells, while the sub-chronic study in rats was the first *in vivo* experiment to examine the regulation of the FR and RFC transporter proteins following ethanol exposure. We demonstrate that the expression of the folate transporter proteins increases in both human and rat PT cells as a result of repeated ethanol exposure. This increase in expression probably occurs as a response to the ethanol-mediated decrease in the PT reabsorption of folate, i.e., an increased amount of the FR and RFC transporters compensates for the reduced folate levels, in an effort to return the body to a state of folate homeostasis.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DME), Ham's F-12 Medium (F-12), Penicillin-Streptomycin (Pen-Strep), Amphotericin B (Fungizone®), Trypsin-EDTA (0.05:0.02%), Fetal Bovine Serum (FBS), L-glutamine, Phosphate Buffered Saline (PBS) and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Purified bovine collagen type I (3.1 mg/ml) was purchased from Vitrogen (Palo Alto, CA). The media supplements insulin, transferrin, selenium, hydrocortisone, and epidermal growth factor were purchased from Becton Dickinson Collaborative Biomedical Products (Bedford, MA). An additional supplement, triiodothyronine was purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals, unless otherwise noted in the text, were purchased from Sigma Chemical Company (St. Louis, MO).

Preparation of Primary Cultures and General Cell Culture Techniques

HPT cells were obtained as normal cortical renal tissue specimens as a result of biopsy or nephrectomy due to tumor or trauma (Urology Department, LSU Health Sciences Center, Shreveport, LA). In all cases, kidney specimens were judged to be normal by the pathologist prior

to use as an HPT cell culture source. As the patient records were not retained and the tissue obtained was regarded as waste for discarding, the Institutional Review Board for Human Research (Louisiana State University Health Sciences Center-Shreveport) exempted these studies. Cortical tissue was isolated by gross dissection, placed in ice-cold HBSS, sliced to form a paste and centrifuged at 913 x g for 30 sec. The tissue pellet was digested in a trypsinizing flask containing 25 ml of a DNAase (400-800 U/ml)-collagenase (>175 U/ml) solution at 37°C for 15 min. The product was filtered through a sterile Nitex filter (Martin Supply Company, Inc., Baltimore, MD) to collect undigested tissue. The filtrate was centrifuged at 228 x g for 10 min and the resulting pellet was resuspended in HBSS. Digestions were repeated 2-3 times and the pellet suspensions were pooled and centrifuged at 228 x g for 10 min. The final pellet was resuspended in DME (containing 15% FBS), seeded onto collagen-coated tissue culture flasks (75 cm^2), and incubated at 37°C with a humidified atmosphere containing 5% CO_2-95% O_2 for two days. Then, the seeding growth media was replaced with normal growth media, a serum-free media to select for proximal tubule cells rather than fibroblasts (Detrisac et al., 1984) (DME/F-12,50:50 mixture by volume, with the following additions: insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), epidermal growth factor (10 ng/ml), triiodothyronine (4 pg/ml), L-glutamine (2 mM), Penicillin-Streptomycin and Amphotericin B). Flasks were fed fresh media every third day and, once established as a stable primary culture, the isolate was frozen in liquid nitrogen or subcultured for a maximum of 8 passages. Every 5-7 days (when the monolayer reached confluency), cells were subcultured by trypsinization and seeded onto new flasks or tissue culture inserts, and grown in normal serum-free growth media for experimentation. Confluency was determined using light microscopic visualization for the flasks and the development of transepithelial resistance (TER) for the inserts (Morshed and McMartin, 1995). TER is indicative of functional tight junctions between HPT cells, and was measured using an EVOM Epithelial Voltohmmeter (World Precision Instruments, New Haven, CT).

Folate Transport Studies in HPT Cells on Inserts

Characterization of 5-methyltetrahydrofolate handling by HPT cells was determined by binding and transport experiments. Transport is a collective term for the cellular uptake and transmembrane transfer across either the apical or basolateral membrane. Confluent cell monolayers were subcultured on collagen-coated Millicell® PCF microporous membrane inserts (0.4 micron,

12 mm diameter, Millipore, Bedford, MA), which were positioned inside the wells of a 12-well tissue culture plate. The advantage of using inserts is that it is possible to isolate the apical and basolateral surfaces of the HPT cells for transport studies (Morshed and McMartin, 1997). Once cell layers were confluent, growth media was removed and inserts were washed thrice, on both the apical and basolateral sides, with pH 7.4 incubation buffer (107 mM NaCl; 5.3 mM KCl; 1.9 mM $CaCl_2$; 1.0 mM $MgCl_2$; 26.2 mM $NaHCO_3$; 7.0 mM D-glucose; 20 mM HEPES) at 37°C before transfer from 12-well to 24-well tissue culture plates. Fresh incubation buffer was added (0.27 ml per insert and 0.6 ml per well) to the new plates, and the inserts were preincubated in the buffer for 60 min to allow for recovery of TER (Morshed and McMartin, 1995). After the preincubation, [^{3}H]-5-methyltetrahydrofolate (25 nM, Moravek Biochemical, Brea, CA) and [^{14}C]-inulin (0.1 mCi/ml final concentration, Amersham Pharmacia Biotech, Piscataway, NJ), with or without unlabeled 5-methyltetrahydrofolate (a 1000-fold excess for nonspecific controls), were added in a combined volume of 30 µl to each insert to measure transport from the apical compartment. Incubations were then conducted for 2 hr at 37°C. Similarly, incubations were conducted from the basolateral compartment by adding substrate to the buffer in the well (outside the insert). Inserts measuring total binding and transport were conducted in quadruplicate, while those measuring nonspecific binding and transport were conducted in duplicate. Specific binding and transport were calculated by subtracting the nonspecific binding and transport values from the total binding and transport values.

After incubation, samples were collected from the apical and basolateral compartments, and inserts were washed thrice on both sides with pH 7.4 buffer to remove nonspecifically-bound [^{3}H]-5-methyltetrahydrofolate. The binding compartment represented what was bound to the FR on the apical membrane and was determined by removing ligand from the receptor with a 30 sec incubation of the apical surface with a pH 3.0 buffer (McMartin et al., 1992). The transport compartment represented what has been transported into the cells and was determined by solubilizing the cells using 0.1% Triton X-100 in PBS. The content of [^{3}H]-5-methyltetrahydrofolate and [^{14}C]-inulin was determined by counting aliquots from each compartment in vials containing 4 ml of 3a70B™ scintillation cocktail (Research Products International Corporation, Mount Prospect, IL) using a dual-label-programmed liquid scintillation counter. The 5-methyltetrahydrofolate content was normalized using the protein concentration of each individual insert to determine the content in fmol/mg protein.

Protein levels were determined in the solubilized cells using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

To test the specificity of the ethanol effect on folate transport, the effect of ethanol on the uptake of the glucose analog, α-methylglucopyranoside (αMG), by HPT cells was measured. Confluent cultures on HPT cells in 12 well plates were washed as above and incubated with methyl-α-D-[U- ^{14}C] glucopyranoside (1 mM, 0.5 μCi/ml, Amersham Pharmacia), 0.1 μCi/ml [^{3}H]-inulin (measure of nonspecific uptake), and various concentrations of ethanol in pH 7.4 incubation buffer for 1 h at 37°C, after which the cells were washed with wash buffer (4°C) and solubilized with 0.2 N NaOH. The radioactivity of the [^{14}C]-α-MG transported into the cells was determined using a liquid scintillation counter. Values for [^{3}H]-inulin uptake were subtracted from total [^{14}C]-α-MG uptake to determine the specific uptake of [^{14}C]-α-MG.

Ethanol Effects on Folate Binding and Transport in HPT cells

Various concentrations of ethanol (50-500 mg/dl) were used to pre-treat HPT cells prior to [^{3}H]-5-methyltetrahydrofolate binding and transport studies. These ethanol concentrations correspond to 11-109 mM, respectively. For the 1 hr treatment, ethanol was added to pH 7.4 incubation buffer to achieve the desired concentrations during the pre-incubation to restore TER. These concentrations of ethanol do not affect tight junctional status, as TER recovered at similar rates in ethanol-containing and control inserts. At the end of the pre-incubation, [^{3}H]-5-methyltetrahydrofolate was added to the cells for the 2 hr binding and transport incubation period, during which ethanol remained in the incubation buffer. For longer treatment periods, ethanol was added to the normal growth media (rather than to pH 7.4 incubation buffer), in order for the cells to maintain the proper nutritional status during the extended pre-treatment periods. In these studies, the ethanol-containing media was replaced daily to minimize depletion of ethanol levels. On the day of the [^{3}H]-5-methyltetrahydrofolate binding and transport incubation, the ethanol-containing media was removed and cells were washed as above to remove the growth media. The cells were pre-incubated for 1 hr in ethanol-containing pH 7.4 incubation buffer prior to the addition of [^{3}H]-5-methyltetrahydrofolate for the 2 hr incubation.

Repeated Ethanol Studies in HPT Cells Cultured in Flasks

To test the effects of repeated ethanol exposure on the content of the folate transport proteins in kidney cells *in vitro,* confluent HPT cell monolayers, in 75 cm^2 flasks, were treated with ethanol in normal growth media for 5 days (repeated) in the following concentrations: 100, 300, and 500 mg/dl. In these studies, flasks were sealed in containers which included a petri dish of media containing the same concentration of ethanol as in its respective flask. The containers were exposed to the humidified atmosphere (5% CO_2-95% O_2) for 30 min prior to being sealed. The media in the flask and petri dish were replaced daily with fresh media to maintain the ethanol content. For the control flasks (0 mg/dl ethanol), normal growth media was added. Prior to the daily replacement of the spent media, a 1 ml volume was removed from the flasks for determination of ethanol concentration by gas chromatography (GC). The ethanol-containing media was stored in microcentrifuge tubes at 4°C until GC analysis using isopropanol as the internal standard (Baker et al., 1969).

At the end of the treatment, the spent media was removed, and the cell monolayer was washed twice with PBS. Five ml of sucrose buffer (0.25 M sucrose, 10 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.4) containing a protease inhibitor cocktail [1 mM para-aminoethylbenzenesulfonyl fluoride (AEBSF), serine protease inhibitor; 100 μM leupeptin, serine and cysteine protease inhibitor; 10 μM bestatin, metalloprotease inhibitor] was added to each flask before scraping cells from the flask. Scraped cells were homogenized using a hand-held glass homogenizer and plasma membranes were isolated by centrifugation (30 min at 30,000 x g, 4°C). Pellets were resuspended in wash buffer (10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) containing protease inhibitor cocktail, and then measured for protein concentration (BCA™ Protein Assay, Pierce, Rockford, IL). The cell membrane samples were stored at -80°C until their subsequent use in gel electrophoresis.

Sub-chronic Ethanol Studies in Rats Using Liquid Diets

To test the effects of repeated ethanol exposure on the content of the folate transport proteins in kidney cells *in vivo,* male Sprague Dawley rats (250-300 g, Harlan, Houston, TX) were acclimated to control liquid diets for 4 d, then received either a nutritionally complete control or ethanol-containing (5%) liquid diet for 14 days (n=8 per group). Each control animal was fed its diet in a pair-fed manner to a corresponding ethanol rat (restricted to the previous day's consumption of its

pair) in order to maintain consistent nutrient intake between groups. Diets were obtained from Dyets, Inc. (Bethlehem, PA) and were administered in a manner and with a complete composition that has been previously described (McMartin et al., 1989; Yamada et al., 1985). The ethanol diets had an energy distribution of 35% fat, 18% protein, 11% carbohydrate and 36% ethanol, while the control diets contained 47% carbohydrate in place of the ethanol. Both diets contained prescribed levels of folate (0.5 mg/l) and did not contain succinylsulfathiazole (which decreases intestinal bacterial synthesis of folate). On day 13, the animals were housed overnight in metabolic chambers, and urine was collected every 8 h in centrifuge tubes that contained β-mercaptoethanol (50 µl per 1 ml urine) and were covered with foil to minimize oxidative breakdown of urinary folates. The 3 samples were combined for a 24 h sample, which was stored at -20°C until analysis for ethanol concentration (GC assay) and for folate content by *Lactobacillus casei* microtiter growth assay, (Horne, 1997). The rats were returned to their home cages for the 14th d.

On day 15, the rats were anesthetized and their kidneys were rapidly excised, decapsulated, and the cortices were removed. The rats were then euthanized. Preparation of the renal apical (BBM) membrane fractions was adapted from previous studies (Antony et al., 1981; Bhandari et al., 1988). The cortices were homogenized in 25 vol ice-cold sucrose buffer (0.25 M sucrose, 10 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.0) containing the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, 1 mM), using a Tekmar Tissuemizer (Cincinnati, OH), then centrifuged at 2,000 x g for 10 min at 4°C. PMSF (1 mM) was added to the supernatant, which was retained for further centrifugation (30,000 x g for 30 min at 4°C). The final pellet was washed three times in 5 ml wash buffer (10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) and resuspended in wash buffer containing the protease inhibitor cocktail mentioned above (1 mM AEBSF, 100 µM leupeptin, 10 µM bestatin). Protein estimation was performed (BCA™ Protein Assay, Pierce, Rockford, IL) on the BBM samples. Finally, the BBM samples were stored at -20°C until their subsequent use in gel electrophoresis.

The animal protocols were approved by the Institutional Animal Care Committee (LSUHSC-Shreveport) and were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Gel Electrophoresis

An aliquot of each membrane protein sample was added to an equal volume of 2X Laemmli's Sample Buffer [1X concentration of 63 mM Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.0025% bromophenol blue]. Protein samples (10 μg for RFC gels and 20 μg for FR gels) were loaded into pre-cast 4-20% Tris-Glycine gels (1.5 mm x 10 well, Invitrogen, Carlsbad, CA), which were placed in the Xcell SureLock™ Electrophoresis Cell unit (Novex/Invitrogen, Carlsbad, CA). The electrophoresis buffer consisted of 192 mM glycine, 0.1% SDS, and 25 mM Tris, pH 8.3 and runs were performed for 2 hours at a constant supply of 125V.

Western Immunoblotting

The protein was transferred from the gels to polyvinylidene difluoride (PVDF) membranes (0.45 μm pore size; Millipore, Bedford, MA) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). Proteins were transferred for 1 hr at 90V in Towbin's Transfer Buffer (12 mM Tris, 96 mM glycine, 20% methanol). Then, nonspecific protein was blocked on the membranes via overnight incubation in 5% bovine serum albumin (BSA) in TBST (Tris-buffered saline, 0.05% Tween™ 20) at room temperature. Membranes were then probed with primary (1°) antibodies, either a polyclonal antibody for the FR or a polyclonal antibody to the human RFC, for 1 hr at room temperature. The polyclonal antibody to the FR was raised in rabbits against the folate binding protein purified from the rat placenta and was a gift from Dr. Sheldon Rothenberg (Suny Downstate Medical Center, Brooklyn, NY). The polyclonal antibody to the human RFC was raised in rabbits using a purified GST-RFC (glutathione S-transferase-RFC) fusion protein as antigen. Anti-GST-RFC antiserum was a gift from Dr. Larry Matherly (Karmanos Cancer Institute, Detroit, MI). After probing with 1° antibody, membranes were washed repeatedly in TBST prior to incubating in the secondary (2°) antibodies for 1 hr each at room temperature. The 2° antibody used first was ImmunoPure® Biotinylated rabbit IgG (Pierce, Rockford, IL). Following the Biotinylated IgG probe, the membranes were washed repeatedly in TBST at room temperature and then incubated with the other 2° antibody, NeutrAvidin™, horseradish peroxidase-conjugated (Pierce, Rockford, IL). After probing with the NeutrAvidin™, membranes were washed extensively in TBST and treated with enhanced chemiluminescence (ECL™) reagents (Amersham Pharmacia, Piscataway, NJ) for the detection of the HRP-labeled 2° antibody. ECL™-treated membranes were exposed to Hyperfilm™

film (Amersham Pharmacia, Piscataway, NJ) and the various band intensities were analyzed using Bio-Rad Quantity One® Version 4.1 Quantitation Software and the Bio-Rad Gel Doc imaging device.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism® version 3.0 software. All folate transporter densitometry data were expressed as optical density units and were normalized to control, with 0 mg/dl ethanol concentration (cells) or control diet (rats) as the control. The statistical methods used for the HPT cell studies were the one-way ANOVA and Tukey's Multiple Comparison Test as the post test, with $P<0.05$ being the level of significance. The statistical method used in the rat diet study was the Student's t-test, with $p<0.05$ being the level of significance. Values cited in the text represent the mean ± SEM.

RESULTS

Inhibition of 5-Methyltetrahydrofolate Transport in HPT cells by ethanol

The treatment of insert-grown HPT cells with ethanol for 1 h in pH 7.4 incubation buffer, prior to apically added 25 nM [^{3}H]-5-methyltetrahydrofolate, had no effect on apical binding as compared to control (Figure 1). However, ethanol at 300, 400, and 500 mg/dL did cause a significant reduction (15, 20, and 26%, respectively) in the apically-directed cellular transport of 5-methyltetrahydrofolate (Figure 1). Similar ethanol pretreatment did not alter the basolaterally-directed cellular transport of 5-methyltetrahydrofolate (Figure 2). Acute ethanol treatment, at concentrations ≤ 400 mg/dL, did not alter the apical uptake of αMG by HPT cells, although there was a significant decrease in uptake at 500 mg/dL (Figure 2). Apical binding and cellular transport of 25 nM [^{3}H]-5-methyltetrahydrofolate, in HPT cells that had been pre-treated with ethanol for 5 days, were not significantly altered at any concentration of ethanol as compared to control (Figure 3). The protein content of HPT cell cultures treated with ethanol for 5 days was significantly decreased by 24, 27, and 30% at 300, 400, and 500 mg/dl, respectively as compared to control. When total folate binding and apical transport per well were computed, without normalizing the data to amount of protein per insert, ethanol at 400 and 500 mg/dl caused a significant decrease in apical binding of 5-methyltetrahydrofolate by 22 and 34% and at 300, 400, and 500 mg/dl produced a

decrease in apically-directed cellular transport of 5-methyltetrahydrofolate by 24, 31, and 36% (Figure 4), a magnitude similar to the reduction in cellular protein. Despite the apparent loss of cells in these repeated ethanol studies, ethanol per se did not induce cell death. In one HPT isolate at the end of the 5 day exposure, the cells were treated with ethidium homodimer, a dye that is taken up only by cells with damaged membranes and is used as a measure of cytotoxicity (McMartin & Cenac, 2000). No increases in ethidium homodimer fluorescence in any group were noted, indicating that ethanol at concentrations up to 500 mg/dl did not affect cellular viability.

HPT Cells Exposed to Repeated Ethanol

The reduction in cell protein content in insert-grown cells during the 5-day ethanol study resulted in modifications to the handling of HPT cells during the repeated ethanol experiment in flasks. To perform the Western blotting optimally without the apparent loss of cells, the studies in flasks were conducted with minimal disturbance of the cell monolayer. The flasks were handled gently and the daily replacement of media was not added directly onto the cell monolayer. The HPT cell handling for the repeated ethanol studies was appropriate, as microscopic visualization of the cell monolayer in the flasks (including 500 mg/dL) on day 5 revealed a confluent monolayer with no apparent cell loss. That there was no apparent cell loss in these studies was confirmed in that the mean total protein content was not different across ethanol concentrations: 1.6 ± 0.2, 1.6 ± 0.2, 1.4 ± 0.1, and 1.2 ± 0.1 mg/ml for 0, 100, 300, and 500 mg/dl ethanol, respectively.

HPT cell isolates were cultured in flasks in media containing ethanol (0, 100, 300 and 500 mg/dl) for a period of five days as an *in vitro* model of the effects of repeated ethanol on expression of folate transporter proteins. The ethanol concentrations in the spent media (removed from the flasks after 24 hr) were determined via gas chromatography and were consistent with the target concentrations of 0, 100, 300, and 500 mg/dl (data not shown). This result indicated that the HPT cells were exposed to a constant level of ethanol during each 24-hr period of the 5-day treatment. Repeated ethanol exposure resulted in an upregulation of the FR and RFC transporter proteins that was dose-dependent on the ethanol concentration (Figure 5). The representative Western blots are expressed as optical density units normalized to control, with control being the values for the 0 mg/dl ethanol concentration. This upregulation in protein expression was greater for the RFC transporter than for the FR transporter protein. The FR and RFC transporter proteins were significantly

upregulated with increasing ethanol concentrations, when data from all isolates were combined ($P<0.001$, Figure 6).

Sub-Chronic Ethanol Experiment in Rats

As an *in vivo* model of the effects of sub-chronic ethanol on the regulation of the folate transporter proteins, a liquid diet feeding study was conducted in rats receiving either a nutritionally complete control or ethanol liquid diet for 14 days. On day 13, the average urine ethanol concentration was 181 ± 40 mg/dl (Table 1). One animal had an extremely low urine ethanol concentration (9.5 mg/dl), which was attributed to this animal consuming only 2 g of diet overnight, thus resulting in a urine volume of only 9 ml. The average urine folate concentration for the ethanol rats was 9.0 ± 2.3 pmol per 24 hr, which was significantly greater than that for the control animals, 2.5 ± 0.5 pmol per 24 hr ($p<0.05$, Student's t-test). On day 15, the kidneys were excised and homogenized to prepare the apical membrane fraction, which was subjected to Western blotting for analysis of folate transporter content. The FR and RFC transporter proteins were significantly upregulated as a result of sub-chronic ethanol exposure, $p<0.01$ (Figure 7). The representative Western blots are expressed as optical density units normalized to control, with control being the values for the rats receiving the control liquid diet.

DISCUSSION

Although ethanol-induced folate deficiency can result from inadequate dietary intake, decreased absorption, and altered hepatic metabolism, increased urinary excretion of folate is a major cause of folate deficiency (Weir et al., 1985). Chronic alcoholism as well as acute ethanol ingestion may result in decreased serum levels and increased urinary excretion of folate. Previous studies in the perfused rat kidney (IPRK) have demonstrated that ethanol directly blocks the renal reabsorption of folate, leading to increased excretion (Muldoon and McMartin, 1994). Using HPT cells cultured in inserts, we have now demonstrated that 1 hr pre-treatment with ethanol (≥300 mg/dl) significantly decreased apically-mediated cellular transport of 5-methyltetrahydrofolate, without affecting the basolateral transport. This transport would represent that which is involved in the reabsorption of folate in the PT. The effective concentrations of ethanol were similar to those that induced increased folate excretion in the IPRK and in vivo (McMartin, 1986).These studies have therefore confirmed

that ethanol directly affects the transport of 5-methyltetrahydrofolate by the renal PT cell, which leads to higher concentrations of folate in the lumen and eventually in the urinary fluid. Presumably, ethanol must affect the uptake of folate by either the FR or the RFC pathways, as these pathways have been shown to carry out the apically-mediated transport in the PT cell (Morshed et al., 1997; Sikka and McMartin, 1998). The present studies have also shown that repeated exposure of PT cells, both *in vivo* and *in vitro*, to folate-depleting concentrations of ethanol leads to an apparent up-regulation of the folate transport proteins.

In contrast to acute ethanol treatment, 5 day pre-treatment with ethanol produced variable effects on 5-methyltetrahydrofolate cellular transport, with no effect at a concentration of 500 mg/dl when data were normally for cellular protein content, yet a significant decrease at concentrations $\geq$300 mg/dl when these data were expressed as total uptake per well. This apparent dichotomy may have resulted from a loss of cells from the membrane during washes of long-term ethanol-treated cells or to increased susceptibility to detachment from the collagen-coated surface after prolonged exposure to ethanol, either way leading to decreased protein concentrations. The loss of protein could explain the diminished effect of ethanol on folate uptake, when compared to control cells with normal levels of protein. When the data were analyzed prior to normalizing the data to protein content, a significant effect of ethanol was observed. An alternative explanation for the lesser effect of ethanol on folate transport by HPT cells after 5 days of treatment, compared to the significant decrease in transport after 1 hr, might be the significant increase in folate transport protein content (both FR and RFC) that we demonstrated after 5 days of ethanol exposure. The increase in folate transporters might be expected to somewhat counteract the inhibitory effects of acute ethanol on folate transport, which could explain the lesser overall effect on transport seen with repeated exposure.

The results obtained from the Western blotting studies in the HPT isolates exposed to repeated ethanol suggest that the decrease in protein content observed in the previous repeated ethanol studies is attributable to the mishandling of the cells. With a gentler handling of the cultures as in the Western blotting studies, there was no apparent cell loss observed via microscopic visualization or by measurement of total protein content.

The excess consumption of ethanol is associated with an increased urinary excretion of folate

(McMartin et al., 1986; Tamura and Halsted, 1983; Russell et al., 1983). Studies with rats have demonstrated that the effect of ethanol on folate excretion is related to the dose of ethanol (McMartin et al., 1989). In the current studies, rats were exposed to sub-chronic ethanol (5%) in a liquid diet for 2 weeks. The urine ethanol concentrations of nearly all rats receiving the ethanol diet corresponded to levels of intoxication. The urine folate concentrations revealed that the sub-chronic ethanol treatment resulted in a modest increase in urinary excretion of folate, similar to that reported in previous diet studies (McMartin et al., 1989), but less than that observed after acute ethanol ingestion (McMartin, 1984). This sub-chronic study in rats was the first *in vivo* experiment to examine the regulation of the FR and RFC transporter proteins following ethanol exposure. The rat *in vivo* study confirmed the *in vitro* studies using HPT cell isolates, in that both folate transporter proteins were significantly upregulated following ethanol administration, whether this exposure was repeated or sub-chronic.

Prior studies in rats have demonstrated that urinary folate excretion is markedly increased 6-fold after acute ethanol exposure (1 g/kg/h for 6 h) and 2-fold after chronic (12 wk) ethanol ingestion in the diet (Eisenga et al., 1989b; McMartin et al., 1989). In the present studies, exposure of rats to sub-chronic ethanol in the diet for 2 weeks resulted in a 3-fold increase in urinary folate excretion. The upregulation of folate transport proteins was apparent following the 2-week period of ethanol ingestion. The upregulation would counteract the decrease in folate transport, since the number of folate transporters would increase to maintain the supply of cellular folates. The upregulation of folate transporters following chronic ethanol exposure would explain the lesser effect in previous rat studies, in which chronic ethanol produces only a 2-fold increase in excretion rather than the 6-fold increase in excretion observed with acute ethanol ingestion.

Similarly, in cultured HPT cells, our studies have demonstrated that acute ethanol decreases 5-methyltetrahydrofolate transport in the apical to basolateral direction. However, when the HPT cells are exposed to ethanol for a 5-day period, the cellular transport of 5-methyltetrahydrofolate does not decrease (on a per mg protein basis). This effect possibly results from an upregulation of the folate transporters following repeated ethanol exposure, which would counteract the decrease in transport observed during the acute ethanol studies.

The FR and RFC transporter proteins were both significantly upregulated when exposed to

conditions of repeated ethanol. For the FR transporter protein, the expression in the HPT cells was significantly increased 1.7-fold and 2.2-fold with 300 and 500 mg/dl ethanol, respectively. In the HPT cells, the expression of the RFC transporter protein was significantly increased 2.5-fold and 4.5-fold with 300 and 500 mg/dl, respectively. Ethanol therefore appears to have a modulating effect on the RFC transporter that is greater than its effect on the FR transporter.

In the current ethanol studies, the significant affects of ethanol in upregulating the folate transporters occurred at ethanol concentrations $\geq$ 300 mg/dl. In previous studies, in which rats or humans are exposed to acute ethanol, a dose of 1 g/kg/h produces blood ethanol levels around 100 mg/dl, and this dose of ethanol does not alter urinary folate levels (McMartin et al., 1986). However, when this dose is increased in rats to 2 g/kg/h, the blood ethanol levels increase to 200-250 mg/dl, and urinary folate excretion increases with a subsequent decrease in plasma folate levels. In the repeated ethanol studies, upregulation of the folate transporters at a concentration of 100 mg/dl was minimal, and statistically significant results were only observed for ethanol levels = 300 mg/dl. These data support the suggestion that the upregulation may be a response to the acute decrease in folate transport.

The various HPT cell isolates examined in these repeated ethanol studies produced relatively consistent results. As each isolate represents normal human kidney tissue discarded as waste from four individual patients, there is a vast potential for inter-individual variability. However, in all four isolates exposed to repeated ethanol, the protein expression for the RFC was increased to a larger extent (almost double) than that for the FR. The concentration effect was similar for all isolates, and when data from all isolates were combined, ethanol concentrations of 300 and 500 mg/dl significantly upregulated both folate transporters.

The larger increase in expression observed for the RFC transporter protein, as compared to the FR protein, was also demonstrated to occur for the *in vivo* model. The protein expression of the RFC transporter in rats administered sub-chronic ethanol (5%) for 2 weeks increased 1.3-fold compared to control animals, whereas the expression of the FR protein increased 1.2-fold compared to control animals. This upregulation in both folate transporters, although not as pronounced as that observed for the HPT cells, was statistically significant for both proteins. The mean urine ethanol concentration in the rats was 181 $\pm$ 40 mg/dl, which is below the ethanol level of 300 mg/dl in the

HPT cells that was required to produce a significant upregulation in the folate transporter proteins. The lower apparent upregulation observed in the rats may have resulted from the rats being exposed to a lesser concentration of ethanol compared to that in the media of the HPT cells. It is crucial to point out that the increase in protein expression for both the FR and RFC transporters is consistent, with a larger effect observed for the RFC transporter and a similar concentration required for upregulation to occur, for both our *in vitro* and *in vivo* models.

In summary, acute ethanol was discovered to inhibit the transport of 5-methyltetrahydrofolate in cultured HPT cells. In the repeated ethanol studies in HPT cell isolates, increasing concentrations of ethanol resulted in an upregulation of the folate transporters to possibly reverse the ethanol-mediated decrease in the reabsorption of folate. In male Sprague Dawley® rats as an *in vivo* model, the FR and RFC transporter proteins were both significantly upregulated in the rats receiving the ethanol diet. The repeated ethanol studies, conducted *in vitro* and *in vivo*, have revealed that administration of intoxicating levels of ethanol results in a significant upregulation of both the FR and RFC transporter proteins. The ethanol-mediated protein upregulation is probably a compensatory response to counteract the effects of ethanol in inhibiting the reabsorption of folate.

FIGURE LEGENDS

Figure 1. Effect of 1 h ethanol pretreatment on apical binding and transport of 5-methyltetrahydrofolate in HPT cells. Insert-grown HPT cells were pre-incubated in the indicated concentrations of ethanol for 1 h prior to the addition of 25 nM [^{3}H]-5-methyltetrahydrofolate to the apical chamber (insert) for 2 h incubation. Binding and transport samples were collected and analyzed as described in Methods. Data represent specific uptake value (binding or transport), normalized to cellular protein and expressed as % of control (0 ethanol) $\pm$ SEM (n = 5). * Indicates significant difference from control (ANOVA, followed by Tukey's test, $p < 0.05$).

Figure 2. Effect of 1 h ethanol pretreatment on basolateral transport of 5-methyltetrahydrofolate and on apical uptake of αMG in HPT cells. Insert-grown HPT cells were pre-incubated in the indicated concentrations of ethanol for 1 h prior to the addition of 25 nM [^{3}H]-5-methyltetrahydrofolate to the basolateral chamber (in well outside of insert) for 2 h incubation. Folate transport samples were collected and analyzed as described in Methods. 12-well plate-grown HPT cells were incubated in the indicated concentrations of ethanol plus [^{14}C]-αMG and [^{3}H]-inulin for 1 h. Specific αMG uptake was determined as described in Methods. Data represent folate transport and αMG uptake values, normalized to cellular protein and expressed as % of control (0 ethanol) $\pm$ SEM (n = 3). * Indicates significant difference from control (ANOVA, followed by Tukey's test, $p < 0.05$).

Figure 3. Effect of 5 day ethanol pretreatment on specific apical binding and transport (normalized to cellular protein content) of 5-methyltetrahydrofolate in HPT cells. HPT cells were grown on inserts in media containing the indicated concentrations of ethanol for 5 d (with daily changes of media to maintain ethanol concentrations), then washed and pre-incubated in the indicated concentrations of ethanol for 1 h prior to the addition of 25 nM [^{3}H]-5-methyltetrahydrofolate to the apical chamber (insert) for 2 h incubation. Binding and transport samples were collected and analyzed as described in Methods. Data represent specific uptake value (binding or transport), normalized to cellular protein and expressed as % of control (0 ethanol) $\pm$ SEM (n = 5). There were no significant differences among the treatment groups (ANOVA, $p > 0.05$).

Figure 4. Effect of 5 day ethanol pretreatment on total apical binding and transport (per well) of 5-methyltetrahydrofolate in HPT cells. HPT cells were grown on inserts in media containing the indicated concentrations of ethanol for 5 d (with daily changes of media to maintain ethanol

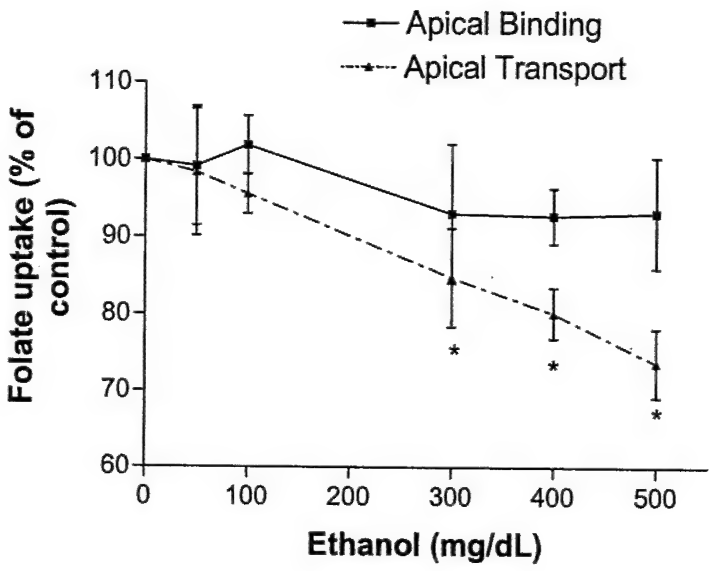
concentrations), then washed and pre-incubated in the indicated concentrations of ethanol for 1 h prior to the addition of 25 nM [^{3}H]-5-methyltetrahydrofolate to the apical chamber (insert) for 2 h incubation. Binding and transport samples were collected and analyzed as described in Methods. Data represent total uptake value (binding or transport) per well in pmol/mL, expressed as % of control (0 ethanol) $\pm$ SEM (n = 5). * Indicates significant difference from control (ANOVA, followed by Tukey's test, $p < 0.05$).

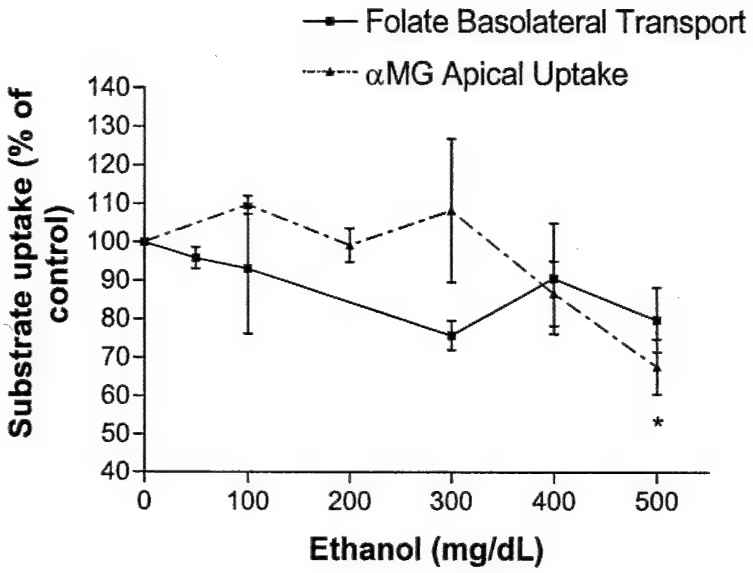
Figure 5. Repeated ethanol exposure dose-dependently upregulates folate transport proteins in individual HPT cell isolates. Western blots (n = 3) were performed on isolated membranes from distinct HPT isolates. Upper blot -RFC protein (83 kD; 10 μg protein per lane); lower blot - FR protein (35 kD; 20 μg protein per lane). The graphs below express the optical density (OD) units of the Western blots, normalized to control (units of the 0 mg/dl ethanol blots). Values represent the group mean ± SEM. A one-way ANOVA revealed significant differences among the ethanol concentrations for both the RFC and FR transporter proteins ($P<0.05$). Bars with no letters in common are statistically different ($P<0.05$, Tukey's Multiple Comparison Test).

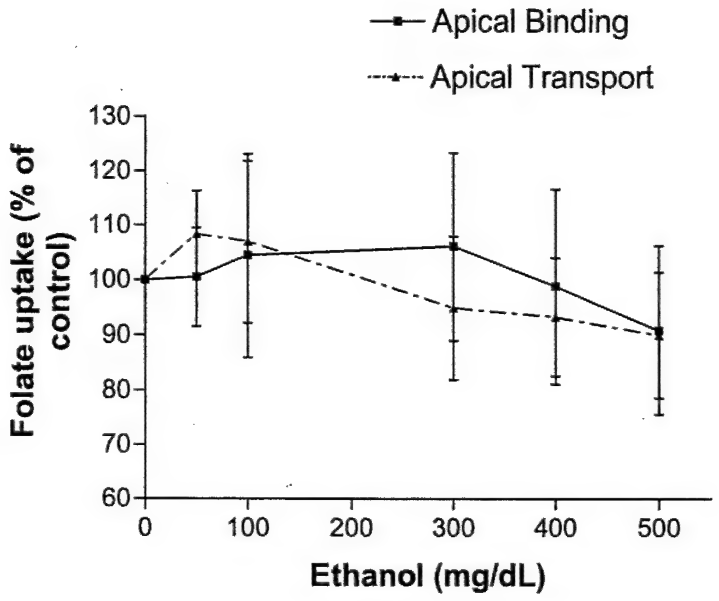
Figure 6. Effects of repeated ethanol on folate transport protein expression in HPT cells. Optical density (OD) units from Western blots of all isolates (n=4) exposed to repeated ethanol were combined and normalized to control (units of the 0 mg/dl ethanol blots). Values represent the group mean ± SEM. A one-way ANOVA revealed significant differences among the ethanol concentrations for both the RFC and FR transporter proteins ($P<0.05$). Bars with no letters in common are statistically different from one another ($P<0.05$, Tukey's Multiple Comparison Test).

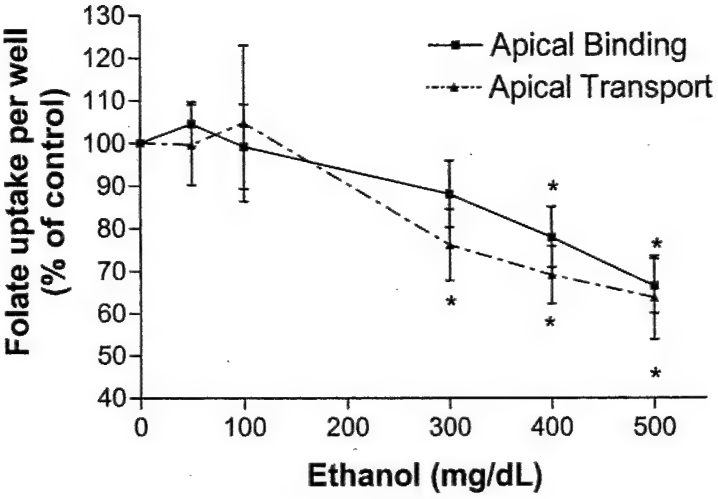
Figure 7. Sub-chronic ethanol upregulates folate transport proteins in rats in vivo. Western blots were performed on the isolated AP membranes from kidneys of rats fed ethanol or control diets for 14 d (n=8 per group). Upper two blots - RFC protein in the 16 rats (83 kD, 10 μg protein per lane); lower two blots - FR protein in the 16 rats (35 kD, 20 μg protein per lane). "C" represents control rats and "E" represents ethanol, with pair-fed rats in adjacent lanes for both the FR and the RFC. The graph below expresses the optical density (OD) units of the Western blots (n=3), in which the OD units were normalized to control (units of the control rats). Values represent the group mean ± SEM. A Student's t-test revealed a significant difference between the rats receiving control and ethanol liquid diets for both

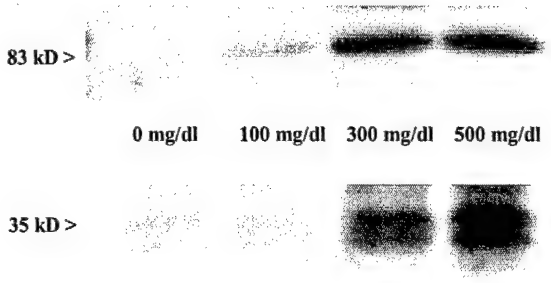
transport proteins ($p<0.01$). ** denotes significantly different, $p<0.01$, compared to control.

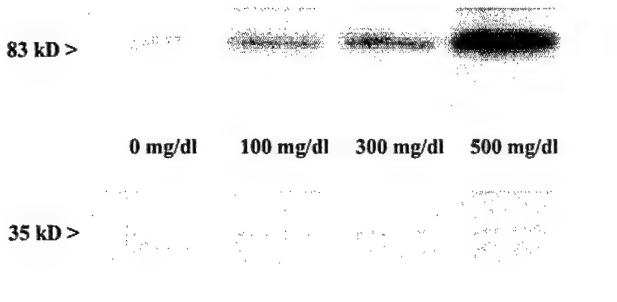

Apical Binding
Apical Transport
Folate uptake (% of control)
110
100
90
80
70
60
0
100
200
300
400
500
*
*
*
Ethanol (mg/dL)

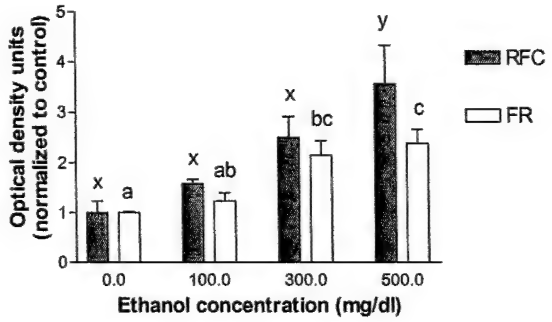

Folate Basolateral Transport
αMG Apical Uptake
Substrate uptake (% of control)
140
130
120
110
100
90
80
70
60
50
40
0
100
200
300
400
500
*
Ethanol (mg/dL)

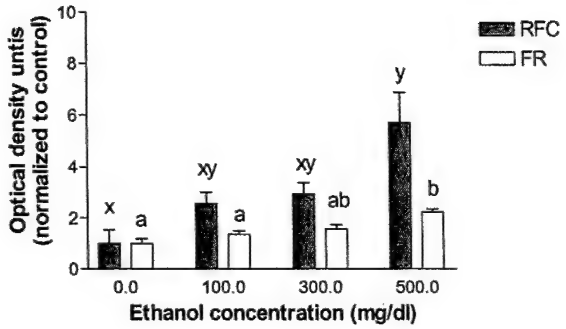

Apical Binding
Apical Transport
Folate uptake (% of control)
130
120
110
100
90
80
70
60
0
100
200
300
400
500
Ethanol (mg/dL)

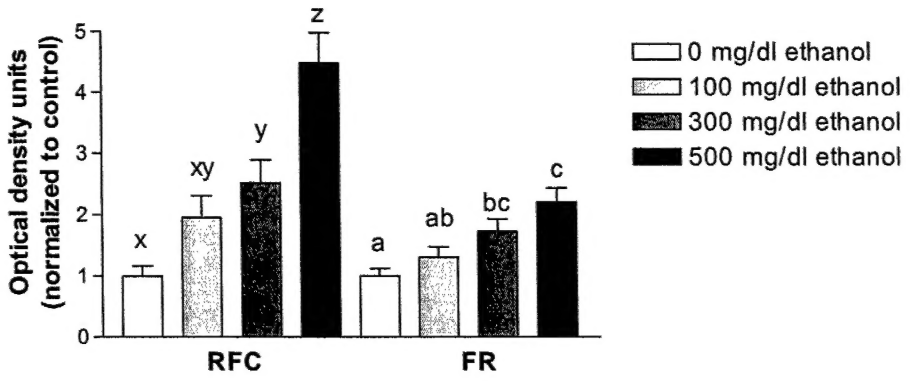

Folate uptake per well
(% of control)
130
120
110
100
90
80
70
60
50
40
0
100
200
300
400
500
Ethanol (mg/dL)
Apical Binding
Apical Transport
*
*
*
*
*

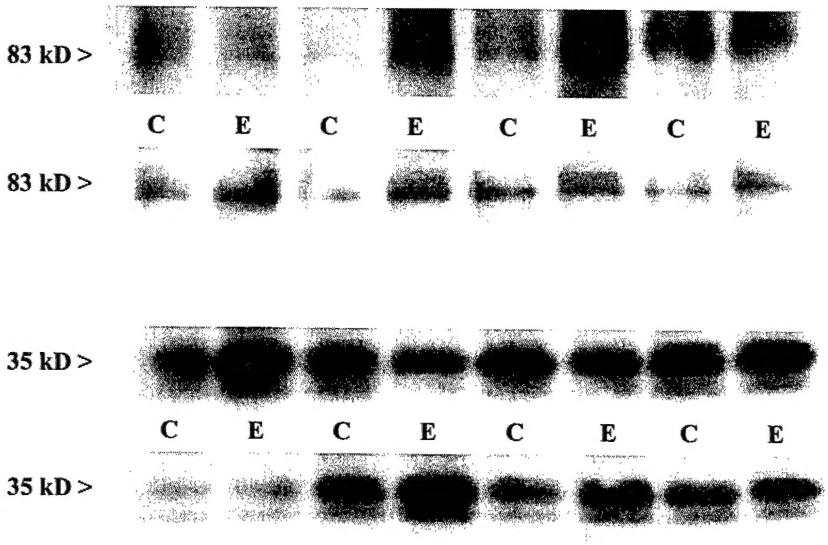

83 kD >
0 mg/dl
100 mg/dl
300 mg/dl
500 mg/dl
35 kD >


83 kD >
0 mg/dl
100 mg/dl
300 mg/dl
500 mg/dl
35 kD >


Optical density units
(normalized to control)
RFC
FR
x
a
x
ab
x
bc
y
c
0.0
100.0
300.0
500.0
Ethanol concentration (mg/dl)


Optical density untis
(normalized to control)
RFC
FR
x
a
xy
a
xy
ab
y
b
0.0
100.0
300.0
500.0
Ethanol concentration (mg/dl)


Optical density units
(normalized to control)
5
4
3
2
1
0
x
xy
y
z
a
ab
bc
c
RFC
FR
0 mg/dl ethanol
100 mg/dl ethanol
300 mg/dl ethanol
500 mg/dl ethanol


83 kD >
C E C E C E C E
83 kD >
35 kD >
C E C E C E C E
35 kD >

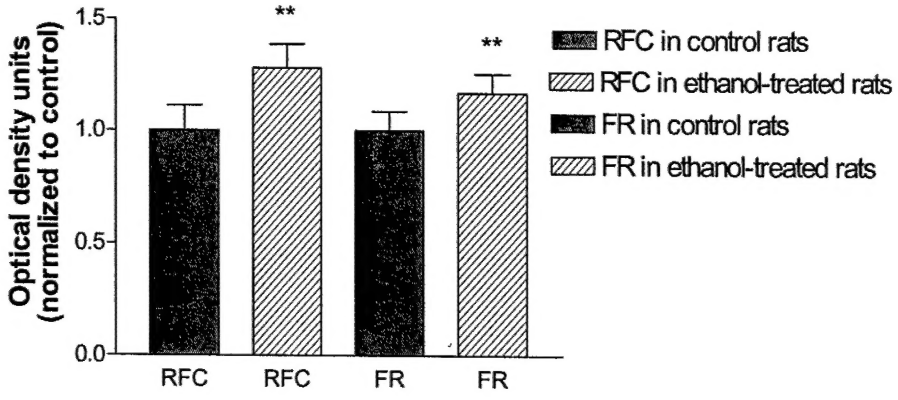

Optical density units (normalized to control)
1.5
1.0
0.5
0.0
**
**
RFC
RFC
FR
FR
RFC in control rats
RFC in ethanol-treated rats
FR in control rats
FR in ethanol-treated rats

Table 1
Ethanol Concentrations in Rats

Animal Number		Urine (mg/dl)
1		9.5
2		141.3
3		250.7
4		99.6
5		239.6
6		115.2
7		215.0
8		375.8

831

ENHANCED DEVELOPMENT OF CHRONIC TOLERANCE TO ETHANOL, CANNABINOID AND MORPHINE IN MICE OVER-EXPRESSING TYPE VII ADENYLYL CYCLASE IN THE BRAIN. P.H. Wu, M. Yoshimura, P.L. Hoffman and B. Tabakoff. Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262

Brain cyclic AMP signaling systems can modulate acute and chronic effects of ethanol, cannabinoids and opiates. To test the hypothesis that a particular adenylyl cyclase (AC) can alter the acute actions and the development of tolerance to these drugs, we examined the effect of overexpressing AC Type VII (AC7) in the mouse brain. AC7 is an isoform of AC whose activity is uniquely enhanced by ethanol, by morphine and by cannabinoid in cell systems containing proper Gs- and Gi- coupled receptors. Mice that overexpress AC7 in the brain (TG) were created using the human AC7 gene controlled by the rat synapsin I gene promoter. Acute and chronic effects of ethanol and morphine in these mice were reported earlier. Now we show effects of cannabinoids. The acute effect and tolerance to the cataleptic action of the CB1 receptor (CB1R) agonist, WIN55212-2 (WIN), was measured by the "ring" immobility test. The membrane CB1R binding of ^{3}H-CP55940 and phospho-CREB (p-CREB) levels in brain of TG and WT mice before and after chronic WIN treatment were measured. Our results showed that TG were more sensitive than WT to the acute cataleptic effect of WIN (ED_{50}: TG, 0.5 ; WT, 1.5 mg/kg). The CB1R was not different between naive WT and TG. A greater tolerance developed in TG mice than in WT mice (the tolerance index: TG, 48; WT, 19). In tolerant mice, the CB1R binding was increased in both WT (from 0.76 to 1.53 pmol/mg) and TG (from 0.74 to 1.25 pmol/mg) with an unchanged Kd. In naive TG mice, an administration of 0.5 mg/kg WIN produced a 37% increase in the striatal p-CREB while a 27% decrease in p-CREB was seen after even an 1.5 mg/kg dose of WIN in WT. In tolerant mice, TG showed an 84% increase in the striatal p-CREB levels while the increase in WT was 23% after treatment with WIN . The measures of acute responses to WIN and to morphine demonstrated that TG (C57/SJL background) were more sensitive than WT mice to certain pharmacological actions of these drugs. With regard to ethanol, however, the TG mice were less sensitive than WT mice. On the other hand, tolerance measures indicated that the TG mice developed greater tolerance to all three drugs. The involvement of AC7 and differential p-CREB generation in the greater development of tolerance in TG mice to all three drugs, will be discussed. Supported by NIAAA and the Banbury Foundation

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The cyclic AMP/protein kinase A signal transduction pathway modulates tolerance to ethanol-induced sedation.
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Departments of Medicine and Psychiatry, The Johns Hopkins University School of Medicine. Baltimore, Maryland, 21205.

Ethanol and other drugs of abuse modulate cAMP/PKA signaling within the mesolimbic reward pathway. To understand the role of the cAMP/PKA signal transduction in mediating certain effects of ethanol, we have studied ethanol-induced sedation in two lines of genetically modified mice. We report that mice with the targeted disruption of one Gsa allele (i.e., knockout) have decreased adenylyl cyclase activity compared to their wild-type littermates. Genetic reduction of cAMP/PKA signaling made these mice more sensitive to the sedative effects of ethanol, even though plasma ethanol concentrations are unaffected. Interestingly, the Gsá knockout mice did not develop tolerance to repeated ethanol challenges whereas their wild type littermates did. In contrast, mice with increased adenylyl cyclase activity resulting from the transgenic expression of a constitutively active form of Gsa in neurons within the forebrain are less sensitive to the sedative effects of ethanol compared to their wild type littermates, even though plasma ethanol concentrations were similar. Tolerance studies in this transgenic line are currently in progress. Thus, the cAMP/PKA signal transduction pathway is critical in modulating sensitivity to the sedative effects of ethanol.

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ETHANOL CONSUMPTION IN ACATALASEMIC, CYTOCHROME P450 KNOCKOUT AND CONTROL MOUSE STRAINS
Van A. Redila, Brian R. Smith and Zalman Amit
Center for Studies in Behavioral Neurobiology, Concordia University, 1455 de Maisonneuve W, H-1013,Montreal, Quebec, Canada. H3G 1M8.

Ethanol consumption was measured in three groups of mice; a group selected for low levels of catalase activity, a cytochrome P450 2E1 knockout (KYP2E1 KO) and a control group. Dr. Richard Deitrich (UCHSC) generously provided the mice strains. The ethanol acquisition studies were conducted at two different times with two samples of mice. For each study twenty-five male mice from each group were group housed 4-5 per cage. Animals were presented with a free choice between ethanol and water on an alternate day schedule, with water only presented on the intervening days. Ethanol was presented in increasing concentrations of 2%, 4%, 6%, 8% and 10% (v/v). Each ethanol concentration was presented for 4 days. Ethanol (10%) consumption was maintained for an additional 20 days. Then, after a 2-week deprivation period animals were represented with a free choice between ethanol (10%) and water for 4 days. Results for both the acquisition and reintroduction phases showed that the acatalasemic group drank significantly more ethanol at the 6, 8 and 10% concentrations for group 1 and at the 10% level for group 2 compared to either the KYP2E1 KO or control groups, although the absolute amount differed between the two runs. The KYP2E1 KO and control group did not significantly differ from each other. Results are similar to a previous study from this lab that showed acatalasemic mice drank more ethanol than control mice did.

B. Lab animal - other

834

834

EVOLUTIONARY ANALYSIS OF A POLYMORPHIC REPEAT IN THE CYP2E1 DISTAL PROMOTER.
S.J. Walker, H.J. Edenberg, K.A. Grant, K.E. Vrana
Centers for the Neurobiological Investigation of Drug Abuse and Neurobehavioral Study of Alcohol Department of Physiology and Pharmacology Wake Forest University School of Medicine, Winston-Salem, NC 27156, and Indiana University, School of Medicine, Indianapolis, IN 46202.

The human ethanol-inducible, nitrosamine-metabolizing enzyme cytochrome P4502E1 (CYP2E1) contains a repeat polymorphism in the 5' regulatory region. The common human allele (CYP2E1*1C) contains 6 and the rare allele (CYP2E1*1D) contains 8 repeats of 42-60 basepairs each. Some reports have suggested a functional relationship between repeat number in this polymorphic region and ethanol consumption. We earlier tested this relationship in a monkey model (Macaca fascicularis) of excessive alcohol consumption and found that cynomolgus monkeys do not contain the repeated motif. The present study is an examination of multiple species of monkey to determine when, in evolutionary development, this CYP2E1 polymorphic repeat arose. Sequence analysis was performed on Cercopithecinae (Old World monkeys), Platyrrhini (New World monkeys) and Strepsirhini (prosimians). The data reveal that the polymorphic repeat arose in Old World monkeys, specifically with Hominidae, as repeat sequences were found in gorilla, pygmy chimpanzee, chimpanzee and humans, but not in spider monkey, pig-tailed macaque, rhesus, nor baboon.
Support provided by P50 AA11997.

2. CELL BIOLOGY

835–845

A. Membrane biology

835–841

835

REPEATED ETHANOL UPREGULATES FOLATE TRANSPORT PROTEINS IN THE KIDNEY
R.L. Romanoff, K.E. McMartin, Louisiana State University Health Sciences Center, Shreveport, LA 71130.

Alcoholism is an important factor in the development of folate deficiency, as chronic alcohol consumption directly alters folate homeostasis in the body via increased urinary folate excretion. There are two folate transport systems located on the apical brush border membrane of proximal tubule cells in cortical nephrons: the reduced folate carrier (RFC) and the folate binding protein (FBP) or folate receptor (FR). Although the mechanisms of ethanol in increasing urinary folate excretion are unknown, we hypothesize that this increased excretion mainly results from decreased reabsorption. In response to the increased excretion and subsequent decrease in serum folate levels, the transporters may upregulate to maintain folate homeostasis. As an in vitro study, human proximal tubule cells were cultured and repeatedly exposed to various ethanol concentrations (0, 100, 300, 500 mg/dl). The cells were homogenized, and the expression of the FR and RFC transporters was determined by Western blotting. As an in vivo study, male Sprague-Dawley rats were administered ethanol in a liquid diet and compared to control animals. The rats were sacrificed, their kidneys removed and homogenized, and the levels of transporter proteins were examined by Western blotting. The in vitro studies revealed that with increasing ethanol concentrations, the expression of both the FR and RFC transporters increased, with a larger effect observed for the RFC protein. The in vivo study revealed that both folate transporters were upregulated in the ethanol-treated rats as compared to controls, with the increased expression being significant for the RFC protein. These results suggest that the ethanol-induced folate deficiency results in an upregulation of the folate transporters to counteract the acute effects of ethanol in decreasing the reabsorption of folate.

836

PROTON-DECOUPLED 31P MR SPECTROSCOPY FOR THE ASSESSMENT OF HEPATIC PHOSPHOLIPID METABOLISM IN PATIENTS WITH ALCOHOLIC LIVER DISEASE
H.P. Schlemmer*; T. Sawatzki*; S. Sammet*; I. Dornacher**; P. Bachert*; G. van Kaick*; H.K. Seitz**
*German Cancer Research Center (DKFZ), D-69120 Heidelberg; **Salem Medical Center, D-69121 Heidelberg.

The pathogenesis of alcoholic liver disease (ALD) is complex and has finally not been clarified. Alterations in the phospholipid composition of biomembranes may be responsible for the development of liver cirrhosis. To assess membrane phospholipid metabolism in vivo, proton-decoupled 31P MR spectroscopic imaging ((1H)-31P SI) was performed in 28 patients with histologically proven ALD and in 13 healthy volunteers. All patients underwent standardized serum analysis, ultrasound imaging, and liver biopsy as part of their initial clinical assessment. Biopsy specimens were scored in non-cirrhosis (NC) and cirrhosis (C) (n=14). (1H)-31P SI was performed with a clinical 1.5-T imager (Magnetom Vision(R); Siemens, Germany) equipped with two radiofrequency systems by using a double-tuned (1H, 31P) planar surface coil. Signal intensity ratios were obtained by integration of fits of resonance lines using software available at the MR scanner. Statistical analysis was done with unpaired t-test. In patients with cirrhotic ALD significantly increased signal intensity ratios of phosphomonoester and phosphodiester, phosphoethanolamine and phosphocholine as well as glycerophosphoethanolamine and glycerophosphocholine were observed ($p<0.01$). These observations indicate alterations in hepatic phospholipid metabolism, which may be associated with chronic alcohol consumption. These data support the hypothesis of an inhibition of phosphatidylethanolamine-N-methyltransferase by chronic ethanol consumption. The results of this study justify the use of polyenylphosphatidylcholine in the treatment of ALD.